BIOCHEMICAL MARKERS OR ENZYME CHANGES THAT MAY PRESAGE THE PRESENCE OF CANCER

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Goal: Our goal was to develop further the uses of gas-liquid chromatography and mass spectrometry instrumentation, under computer management, for the study of derangements of human biochemical metabolism due to neoplastic change; this was done for the purpose of identifying new metabolites, or altered levels of known metabolites, which might be diagnostic of cancer.

Approach: (a) To screen urine by GC/MS from control subjects and from individuals suffering from prostatic cancer, to determine if polyamines are indicators of cancer;(b) the quantitation of urinary protein amino acid and organic levels in cancer patients, and quantitative intercomparison of these levels among patients and controls; (c) the quantitation of urinary beta-aminoisobutyric acid levels from cancer patients.

Progress:

A. Development of an Analytical Method for the Quantitation of Urinary Polyamine Levels.

Our intent in pursuing this study was to determine if the polyamines can be used as markers for the early detection of prostatic cancer. Prostatic cancer was chosen because the highest concentration of polyamines in the human body can be found in the male prostate gland. After the method had been developed, it was applied to clinical analysis. We summarize in Table 1 the samples available for study.

A sensitive and specific method using mass fragmentography for the analysis of the polyamines putrescine, cadaverine, spermidine and spermine was developed, along with a method of synthesis for their deuterated analogs. The procedure involves addition of a known amount of a standard solution of deuterated analogs to the urine, followed by overnight acid hydrolysis, butanol extraction and ion exchange chromatography on a strongly cationic ion exchange resin. These procedures are described in detail in Appendix I. The polyamine extract is trifluoracetylated, and polyamine quantitation is achieved by measuring peak height ratios of specific ions characteristic of the trifluoroacetyl derivatives of the indigenous and deuterated polyamines, m/e 126 and m/e 154 being characteristic of the derivative of the indigenous material, and 128 and 156 being characteristic of the derivative of the deuterated analog.

TABLE I. Summary of Data Available for Polyamine Analysis

Lab. No.	Disease	Age	Urine Volume
229	Ca. Prostate	-	1100 cc
238	Normal	51	1410 cc (12 hr)
239	Normal	70	1840 (12 hr)
302	Ca. Prostate	58	1610 cc
324	Hodgkins	29	632 cc (12 hr)
329	Ca. Prostate Grade II	69	1135 cc
330	Ca. Prostate Grade I	61	2370 cc
336	BPH	56	1300 cc (12 hr)
337	BPH	61	1790 cc
340	BPH	64	1430 cc (12 hr)
343	BPH	-	-
344	BPH	62	990 cc (8 hr)
345	BPH	62	745 cc
347	BPH	71	1250 cc (16 hr)
348	BPH	71	372 cc (8 hr)
349	BPH	51	900 cc (8 hr)
351	BPH	51	590 cc (8 hr)
354	Ca Prostate Grade II	51	3220 cc
356c	BPH	52	620 cc (8 hr)
362	Ca. Prostate Grade II	64	1425 cc
363	Ca. Prostate Grade II	74	1550 cc
1-74	Ca. Prostate Grade II	63	970 cc (12 hr)
2-74	Ca. Prostate Grade III	64	720 cc (12 hr)
3-74	Ca. Prostate Grade III	65	560 cc (12 hr)
4-75	Ca. Prostate Grade III	56	440 cc (12 hr)
5-75	Ca. Prostate Grade I	65	525 cc (12 hr)
6-75	Ca. Prostate Grade III	54	620 cc (16 hr)
7-75	Ca. Prostate	66	407 cc (12 hr)
8-75	Breast Ca.	50	1240 cc
9-75	Ca. Prostate	50	695 cc (12 hr)
10-75	Ca. Prostate	51	2140 cc
11-75	Ca. Prostate	61	475 cc (12 hr)
12-75	Ca. Prostate	?	1050 cc (12 hr)
1	Control	39	837 cc
2	Control	33	1760 cc
3	Control	39	1460 cc
4	Control	35	1758 cc
5	Control	27	930 cc
6	Control	26	1020 cc
7	Control	41	1248 cc
8	Control	43	2030 cc
9	Control	35	1430 cc
10	Control	?	1330 cc

After injection of the samples into the GC/MS system, approximately one-half hour is required for analysis, followed by five minutes for processing the analytical data. The concentrations of indigenous polyamines are expressed in mg/100 ml at the end of the time required for processing. During the actual GC/MS runs, the computer monitors the specific ions (126, 128, 154 and 156) characteristic of the materials being analyzed, and at termination of the runs, the total ion current (Fig. 1) is printed out at a CalComp plotter.

During processing of the analytical data, the contributions of the individual specific ions to the total ion current are separated and displayed on a T.V. Monitor along with background substraction (Fig. II), and the finding of the peak's maximum (Fig. III).

Before the actual urine analysis, several quantitative mixtures of pure deuterated polyamines and pure non-deuterated polyamines are made up and derivatized. These mixtures are analyzed by GC/MS and processed to determine the specific ion ratios for each polyamine. These ratios are then plotted versus relative concentrations of the nondeuterated and deuterated polyamines to establish calibration curves for the individual polyamines. During the actual urine analysis, the calibration curves are used to determine the relative concentration of the non-deuterated indigenous materials, from the specific ion ratios determined. The concentrations of the polyamines in the urine can then be determined.

We have examined the samples summarized in Table I using the above procedure. Further methodological details and a presentation of results are to be found in the attached preprint (Appendix I). Briefly, we found, contrary to previous reports, no significant differences between diseased and normal patients in any of the polyamines analyzed. In our opinion, based on this limited sample size, any further attempt to detect or stage prostatic cancer using urinary polyamine levels is worthless. (See Appendix I for a review of the literature on this topic.)

B. Screening of Urine for Metabolites Which Might Presage the Presence of Cancer.

Each urine is fractionated as described in our original proposal into an acidic + neutral fraction, an amino acid fraction and a sugar fraction. We decided to divide the acidic + neutral fraction into equal portions,one-half being methylated with diazomethane (as described in

the original proposal) and the second half is silylated (BSTFA). The latter derivative was included in our analysis for two reasons. First, diazomethane will cause complications in the derivatization of alpha-keto acids and some heterocyclic systems. This does not occur with silylation and a large number of reference mass spectra of TMS derivatives are available for the identification of unknown compounds from library search routines.

The acidic and neutral fraction was divided into two equal portions, one of which was methylated with diazomethane (D-OME) while the other was silylated with BSTFA + 1% TMCS (D-TMS). The sugar fraction was derivatized to the TMS derivative (S-TMS) with TRI-SIL-Z. The amino acid fraction was also divided into two equal portions with one portion silylated with BSTFA + 1% TMCS (E-TMS) and the other converted to N-TFA-O-n-butyl derivative (E-TAB). Details of the procedure have been presented in previous reports.

Each of the six fractions of each urine was then analyzed by the GC/MS/Computer system. Each fraction yields about 600 complete mass spectra. These spectra are processed by a computer program, called "CLEANUP", which is designed to detect components and remove from the spectrum of each component interference from background, column bleed and overlapping components. This procedure yields spectra which are much more characteristic of the spectra of pure compounds than are the raw data. Each fraction may yield from 30-60 spectra representing a GC/MS "profile" for each fraction for each patient.

We have assembled libraries of mass spectra of known compounds by dividing an available collection of over 3000 spectra of compounds of biological interest into subclasses corresponding to the chemical fractions isolated in the above procedure. The appropriate library is searched for the spectrum of each component detected by CLEANUP. Spectra of components which were not matched to the library are examined further in collaboration with the NIH supported DENDRAL project for computer-assisted structure elucidation.

Quantitative intercomparison of profiles to detect components present in abnormal amounts is carried out by automated computer analysis of the GC/MS profile by the HISLIB program. This program is described in detail in Appendix II.

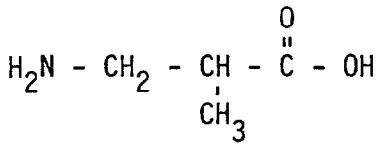
We have examined in detail the urinary profiles from the samples summarized in Table II.

Table II.

TYPE OF CANCER	# OF PATIENTS
Bladder	8
Non-Hodgkin lymphomas	6
Prostatic	5
Leukemia	7
Breast	6
Lung	6
Pancreatic	6
Colon	6

Obviously, with this limited a sample size we depend on detection of either a unique biochemical marker for cancer, or a normal metabolite present in grossly abnormal amounts. The analytical situation is further complicated by the fact that the sample we analyzed were collected from persons under no dietary control and in various stages of illness. Thus, there is no such thing as a "normal" profile of metabolites. In short, all of the problems of drawing definitive answers from urine analysis are present here, and further complicated by artifacts from the collection procedures such as phthalates. We did, however, consider an "average" profile of controls and patients as a basis for detection of novel or abnormal amounts of components (see Appendix II for a description of techniques for comparing GC/MS profiles).

We detected no novel biochemical markers in the group of patients (Table II) or any subgroup which could be used to detect cancer. However, we did note a remarkably high incidence of excretion of β-aminoisobutyric acid (1) throughout the set of samples



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examined. The excretion of 1 is a highly complex problem involving various disease states and genetic factors. The situation is made more complex because not all of the samples analyzed displayed abnormal amounts of 1. Whether this is due to stage of cancer, other illnesses or other factors is unknown. The correlation is present, however, and in the next section we describe work already done and more which might be done in the future to clarify this problem.

C. Quantitation of β-Aminoisobutyric acid (1).

β-Aminoisobutyric acid occurs in significantly elevated amounts in about 70% of the samples studied. It is most prevalent in urines from patients with leukemia (all 7), bladder (in 7 of 8 samples) and lymphomas (5 of 6 samples).

The excretion of 1 has a genetic relationship. D-Beta-aminoisobutyric acid (1) (BAIB) was first identified in normal urine by Crumpler and Dent (1), and shown to be a catabolite of thymine (2,3). BAIB has also been reported to be excreted in a variety of other conditions (4) including cancer (4,5).

The genetic basis for the excretion of BAIB in human urine was first proposed by Harris (6) as a result of a study on a series of families in London. Other studies have shown that in Caucasoid populations, the frequency of high excretors of BAIB is lower than 10% (6,7), whereas in Oriental populations the frequency of high excretors is about 40% (8,9). As a result of family studies, a general hypothesis was put forth, that the homozygote for a recessive allele is a high excretor, and both the homozygote and heterozygote for a dominant allele are low excretors. Experimental support for this hypothesis has been presented by Yanai et al (9) who have shown that the distribution of BAIB concentration in the urine of a Japanese population was bimodal, thereby classifying the population into high and low excretors, the low excretor group being composed of heterozygous and homozygous low excretors. Kakimoto and co-workers (8) isolated and purified the enzyme BAIB:pyruvate aminotransferase, which was found to be present in mammalian livers. This enzyme metabolizes D-BAIB but not the L-isomer. A later publication (10) showed that genetically determined high excretion of BAIB was associated with reduced activity of BAIB:pyruvate aminotransferase. The enzyme from human liver obtained at autopsy was isolated and partially purified. The general character of human liver enzyme was found to be similar to that of hog liver enzyme. The activity of human liver BAIB: pyruvate aminotransferase was low in the high excretors, though not absent.

This study showed as before that the distribution of BAIB in the urine of a Japanese population was bimodal, classifying the population into the high and low excretors, the low excretor group being composed of the heterozygous and homozygous low excretors. The BAIB concentrations were higher in the heterozygous low excretors than the homozygous low excretors, since the heterozygous low excretors are expected to have a lower enzyme activity than that of homozygous low excretors. Tariguchi and co-workers (10) suggested that if the enzyme activity was determined with a larger number of liver specimens, distribution of the activity may be trimodal. In order to demonstrate this phenomenon, they tried to find a more readily available tissue or cell other than liver, but found that human blood cells lacked detectable enzyme activity, even when leukocytes from 20 ml of blood were used.

Current methods of quantitation of BAIB involve either paper chromatography (11), thin layer chromatography (10), electrophoresis (8,9) or ion exchange chromatography (13). These methods are, however, nonspecific for the absolute and unambiguous identification of BAIB. A precise method of quantitation would be highly desirable, especially when one is dealing with low nanogram quantities of BAIB.

The enzyme BAIB:pyruvate aminotransferase catalyzes the reversible reaction

D-BAIB + Pyruvate <—> L-alanine + methylmalonyl semialdehyde

Two methods have been used to measure enzyme activity. The first involves determination of L-alanine formed in the reaction, using an amino acid analyzer (8). The second method involves the use of D-beta-action (-Me-14C) isobutyrate as substrate and measurement of radioactivity in the deaminated product (10). Both methods are non-specific, and the products of the reaction have not been properly characterized.

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We have studied GC/MS methods for quantitation of BAIB (see Reference 1, below); an alternative method utilizing dentenium labelled BAIB would be more precise. Obviously considerable additional work remains to be done in order to establish the significance of high incidence of excretion of BAIB in cancer patients. The question of genetic phenotype needs to be settled for each patient before one can conclude whether or not high excretion is normal.

Publications Relating to the Work

1). W. E. Pereira, R. E. Summons, W. E. Reynolds, T. C. Rindfleisch and A. M. Duffield, "The Quantitation of β-Aminoisobutyric Acid in Urine by Mass Chromatography," Clinica Chimica Acta, 49, 401 (1973).

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5). E.T. Everhart, T.C. Rindfleisch, W.L. Fitch, W.E. Pereira, and A.M. Duffield, "Application of Computerized Stable Isotope Mass Fragmentography to Analysis of Polyamines in Urines of Cancer Patients," submitted for publication (preprint attached).